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This work describes the development of a novel system for efficient and rapid introduction of genes into specific tissues or cells in mice. Detailed analysis of gene function often relies upon introduction of genes into an animal model system. We are developing a retroviral-based system in which controlled expression of the cellular receptor for the virus in a defined pattern determines the site(s) of infection. Using this retroviral gene delivery system, a gene can be introduced not only in a spatial-specific pattern but also in a temporally-controlled manner using a retroviral vector. For these studies we expressed the receptor for the avian virus Rous sarcoma virus in mammary cells in transgenic mice using a MMTV LTR. The ability to selectively target retroviral infection has numerous applications in areas as diverse as developmental biology, gene therapy, and oncogenesis. Here we will attempt to utilize this system for targeted expression of genes in mammary cells to rapidly assess the potential of these genes to induce mammary tumors in an animal model.

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FOREWORD

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INTRODUCTION

The purpose of this project is to develop a novel system for rapidly introducing genes into mammary tissue in an animal model for analysis of gene function in vivo. An important feature of the proposed scheme which distinguishes it from standard transgenic systems is that it allows temporal control of gene expression. This project relies on the ability of a receptor for a retrovirus (Rous sarcoma virus) expressed as a transgene in mammary cells to direct infection of these cells *in vivo*, thus allowing delivery of genes carried by retroviral vectors directly to the mammary cells for rapid assessment of the oncogenic potential of these genes. The goal of this IDEA grant is to prove that this targeted delivery system can work in the mammary gland and to optimize protocols for introduction of genes into mammary epithelial cells in vivo.

The Specific Aims of this research project are:
AIM 1) Characterize transgene expression in mice carrying an MMTV LTR/tva transgene.
AIM 2) Produce high titer MLV(RSV) pseudotyped viruses carrying histochemical markers.
AIM 3) Assay infection of mammary epithelial cells with these viruses and optimize the infection strategy.

BODY

AIM 1 Characterization of the MMTV-tva transgenic mice

When this grant was written we had already successfully established two transgenic lines of mice carrying an MMTV-tva transgene. However this past year problems with mouse hepatitis virus (MHV) infection of our transgenic lines that hampered our production of transgenics for these lines. Because of MHV we were required to move these animals to a different ("dirtier") facility, while we prepared to re-derive the lines by embryo transfer. Unfortunately, at this facility losses due to the MHV infection coupled with poor breeding of our C57BL-6 backcrossed transgenic animals (possibly due to a concurrent pinworm infection) caused both lines #1 and #22 to be lost. Thus, our work this year is to re-establish these mice by re-injecting the same transgene construct into eggs. However, this work is being done at a new strict barrier animal care facility. This facility allows no animals to be imported except by embryo transfer and has experienced no infectious disease outbreaks in its two year existence. Thus we are confident that our transgenic lines will not be

compromised in this facility. Because of the loss of these lines we have not characterized the transgene expression, but have instead focused on developing the vectors and protocols for introducing gene into mammary epithelia in anticipation of the re-establishment of the MMTV-tva mice.

AIM 2. Production of high titer MLV(RSV) pseudotyped vectors carrying histochemical markers.

We proposed use of a transient system to produce MLV viruses carrying the RSV EnvA protein which would allow directed infection of these viruses into cells expressing tva. One advantage of this system over replication competent RSV vectors is that the simpler defective MLV vector backbone allows easier insertion of genes and accommodation of larger inserts. In addition, the RSV vector encodes the structural proteins of this virus and when introduced into mice after the neonatal stage is likely to elicit an immune response, potentially resulting in clearance of infected cells carrying the transduced gene. The defective MLV vectors do not encode any structural genes. To fully take advantage of the transient MLV system we needed to extensively modify the original vector described for use in this system (pHIT110). The original vector was quite large (9.1kb) and contained a G418 resistance gene which we decided to remove to avoid potential immune recognition of infected cells. The plasmid we constructed, pHIT110 poly, is much smaller (5.1kb) and unlike pHIT110, it is derived entirely from known sequences. pHIT110 poly contains a strong cytomengalovirus immediate early enhancer-promoter fused to the R and unique 5' (U5) region of MLV, an extended MLV packaging signal for efficient incorporation of the genomic RNA into virions, a polylinker region with ten unique sites for introducing genes of interest, and the complete 3' MSV LTR for efficient expression of the transduced gene in the infected cells. We have inserted a nuclear localized β-gal gene into pHIT110 poly and produced MLV(VSV-G) pseudotypes. Using this modified vector titers averaging 1 X 10^6 IU/ml were routinely obtained. Thus the re-engineered MLV vector is functional and will allow construction of marker- or onco-gene encoding viruses.

Another feature required for vectors injected into the mammary gland is high titer. This requirement stems from the fact that very small volumes can be injected (maximum of 50μl). To achieve the highest titers possible we would like to concentrate the MLV(envA) viruses. Therefore, we have experimented with two procedures to produce high titer stocks, ultracentrifugation and ultrafiltration. We find that a high level of concentration can be

achieved for EnvA pseudotyped viruses by ultracentrifugation while ultrafiltration results in a loss of infectivity. It appears that the most critical parameter for successful concentration by ultracentrifugation is slow resuspension of the pelleted virions (overnight at 4° without vortexing works best). Thus the protocol routinely employed to concentrate MLV(EnvA) viruses is to harvest media from transiently transfected 293T cells 36 hours after the DNA is added. The media is clarified by low speed centrifugation (3600 rpm for 10 minutes) and then virions are concentrated by centrifugation at 80K X g for 15 minutes. The viral pellet is resuspended in Tris buffered saline overnight at 4°. Using two sequential concentration steps we have achieved a greater than 200-fold increase in MLV(EnvA) β-gal titers as assayed on cultured cells (average final titer $2X10^7$IU/ml on quail QT6 cells). A viral stock of this titer allows injection of $5 X 10^5$ IU of cell-free virus per gland.

Recently, we have found that mammary glands taken from mice during lactation or immediately post-weaning contain significant levels of endogenous β-gal activity. To avoid problems detecting a β-gal marker gene in this setting, we are constructing vectors that carry markers other than β-gal for analysis of *in vivo* infection. One such vector is an MLV genome encoding green fluorescent protein (GFP) or variants of GFP that have been optimized for expression in eukaryotic cells or that have shifted excitation and emission spectra compared to wild type GFP. To avoid potential problems of in vivo immune responses to other proteins encoded by the MLV vectors, we are using the minimal viral vector described above that encodes GFP (pHIT-GFP) but no selectable marker gene. In addition, we have obtained a vector encoding a human alkaline phosphatase from Dusty Miller (pLNCAP). This vector encodes G418 resistance as well as alkaline phosphatase. While the pLNCAP vector will be used in preliminary infection studies optimizing infection parameters (see below), we are currently moving the AP gene into pHIT110 poly to produce pHIT-AP which should avoid immune response problems mentioned above and from our experience should result in higher titer MLV(EnvA) viruses.

AIM 3 *in vivo* infection of mammary epithelial cells

MLV and RSV infection require nuclear envelope breakdown associated with cellular replication, thus we need to develop protocols in which virus is injected when mammary

cells are known to be dividing. Unlike most organs, the mammary gland undergoes extensive growth and development after birth in the juvenile and adult animal. Beginning at approximately 3 weeks of age and continuing through 9 weeks, there is extensive proliferation of ductal cells in the mammary gland. These cells also express the MMTV LTR, so this period should allow efficient infection by RSV in the transgenic mice. Another period of mammary cell proliferation occurs during pregnancy in preparation for lactation. To target cells during this phase of growth, adult (6 month) female mice will be mated, then 14 days after vaginal plugs are noted they will be injected with RSV vectors. The two phases of mammary cell proliferation are not equivalent, so we may target different cells by using these two time frames for infection.

To begin optimizing the parameters for infection of mammary epithelial cells before we re-derive the MMTV-tva mice, we are utilizing MLV vectors carrying envelope proteins that are known to direct infection of a wide variety of cells in culture. This work is in preparation for studies on directed infection with MLV(EnvA) viruses when the MMTV-tva transgenic animals become available. Toward this end, we have begun producing MLV viruses pseudotyped with either vesicular stomatitis virus glycoprotein (VSV-G) or the Ebola Zaire strain glycoprotein (EboGP). Both these envelope proteins mediate infection of a wide variety of cell types in numerous species, thus it is likely that they will allow infection of mammary epithelial cells. We chose to use these envelopes instead of MLV ecotropic envelope (which will also mediate infection of murine cells) because of the stability of VSV-G and EboGP. In both cases viral stocks can be concentrated by centrifugation as described above to produce the titers required for in vivo administration of viruses. This is not the case for the MLV ecotropic envelope protein which is more fragile and does not allow concentration.

MLV(EboGP) and MLV(VSV-G) viruses carrying pMX-GFP or pLNCAP are currently being prepared and concentrated. They will be injected directly into the mammary gland of female mice at the stages of puberty or pregnancy discussed above to determine conditions during which mammary epithelial cells are good targets for retroviral infection in vivo. The optimal conditions determined by these studies will then be employed in future studies with MLV(EnvA) vectors and MMTV-tva mice.

CONCLUSIONS

The work completed this year is very preliminary. However, we have determined conditions for producing EnvA carrying viruses at titers that will be useful for *in vivo* utilization. In addition, we have constructed a CMV promoter-driven MLV vector that will allow construction of marker- or onco-gene expressing vectors for use in vivo. Unlike many available MLV vectors, this vector lacks any other genes that might be immunogenic when injected into adult animals and would therefore complicate analysis of gene transfer into mammary epithelium. Finally, my lab has produced high titer pseudotype viruses carrying broad-tropism envelopes (VSV-G and Ebola GP) that can be used in pilot experiments aimed at defining optimal conditions for infection of mammary epithelial cells.